


# Unveiling Potential Blood Markers for Endometriosis Through the Integration and Experimental Validation of Immune Cell Traits Genome and Genome-Wide Associated Data

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**Background:** While endometriosis (EM) has been previously associated with multiple immune factors, the causal relationship underlying these associations remains unclear.

**Objective:** In this study, Two-sample Mendelian randomization (MR) method was employed to investigate the causal relationship between 731 immune cell traits and EM based on tabulated data from genome-wide association studies (GWAS).

**Methods:** MR method includes inverse variance weighting (IVW), the weighted median (WM), MR-Egger, the weighted model, and the simple model. IVW is used as the primary method for judging causal effects. Peripheral blood was obtained from EM patients, and the positive immune cell phenotype was confirmed using flow cytometry.

**Results:** After *P-value* correction, our two-sample MR showed that *CD28 on CD28+ DN (CD4-CD8-)* had a suggestive causal relationship with EM ( $\beta = 0.040$ , 95% CI = 1.02–1.06,  $P = 0.00029$ ,  $P_{FDR} = 0.1984$ ). The results of the other two main methods were similar: Weighted median (OR = 1.031, 95% CI = 1.00–1.07,  $P = 0.082$ ); MR-Egger (OR = 1.032, 95% CI = 1.10–1.06,  $P = 0.044$ ). The flow cytometry results indicated that the expression level of *CD28 on CD28+ DN (CD4-CD8-)* was significantly increased in the ectopic intima of EM patients.

**Conclusion:** Our study demonstrated a causal relationship between immune traits and EM, and the results were verified by clinical samples. The study may provide new biomarkers for the early diagnosis and immunotherapy of EM.

**Keywords:** endometriosis, Mendelian randomization, immune cell traits, flow cytometry

## Introduction

Endometriosis (EM) is a common oestrogen-dependent inflammatory disease, defined as the presence of functional endometrial glands and stroma outside the uterine cavity.<sup>1</sup> EM frequently results in pelvic pain, abnormal menstrual cycles, and infertility, impacting approximately 10% of women in their reproductive age.<sup>2</sup> The diagnosis of EM includes laparoscopy, transvaginal ultrasound (TVUS), and magnetic resonance imaging (MRI).<sup>3</sup> In the past, it relied too much on the diagnostic of laparoscopic surgery, which has risks of anesthetic, injury, and excessive expense. Meanwhile, some deep or invisible lesions may also be missed due to the heterogeneity in the location and appearance of the endometriotic lesions.<sup>4</sup> Depending solely on laparoscopic surgery to diagnose EM will postpone the diagnosis and treatment of the

condition. Therefore, it is crucial to examine the essential mechanism of EM and discover novel effective diagnostic indicators. In 1927, J. A. Sampson proposed the retrograde menstrual hypothesis of the distant spread of endometrial cells or tissues through blood vessels or lymphatic vessels, which is now widely recognized.<sup>5</sup> With further research, it was found that EM was additionally linked to immunological factors.<sup>6</sup>

The malfunctioning of the immune response is one of the prerequisites that lead to the development of endometrium lesions. Immunological investigations suggest that EM patients experience substantial alterations in both cellular and humoral immune function.<sup>7</sup> A number of aberrant alterations in immunity play crucial roles in the development, proliferation, and invasion of EM cells, including reduced activity of NK cells, increased quantity and cytotoxicity of macrophages, inappropriate activation of T cells and B cells, elevated levels of immune inflammatory components, and production of autoantibodies.<sup>8</sup> A previous study has demonstrated that the percentage of CD8+ T cells, activated NK cells, CD56+ NK cells, and follicular helper T cells was notably elevated in the endometrium of EM patients. Conversely, M2 macrophages, CD163+ macrophages, and resting mast cells were significantly decreased in EM patients when compared to healthy women.<sup>9</sup> While numerous observational studies have demonstrated the correlation between immune traits and EM, there remains a lack of investigation into their causal relationship.

Mendelian randomization (MR), relies on the principles of Mendelian independent distribution laws, identifies genetic variants associated with exposure which regarded as instrumental variables (IVs) to determine the causation. Recently, mounting research have using MR to analyse causal effects which can hardly be determined in observational study due to epidemiological etiologic inference.<sup>10,11</sup> This study employed a comprehensive two-sample MR analysis to establish the causal association between immune cell traits and EM, which was validated in clinical peripheral blood samples.

## Materials and Methods

### Study Design

Based on two-sample MR analysis, we evaluated the causal association between 731 immune cell traits and EM. The IVs utilized in the MR analysis for causal inference must adhere to three fundamental assumptions: (1) the correlation hypothesis: genetic variation is directly correlated with exposure; (2) the independence hypothesis: there is no connection between the genetic variant and the possible confounders between exposure and outcome; (3) the exclusive hypothesis: the genetic variation will not influence the outcome by any methods other than exposure.<sup>12</sup> The complete process is illustrated as a flowchart in [Figure 1](#).

### Data Source

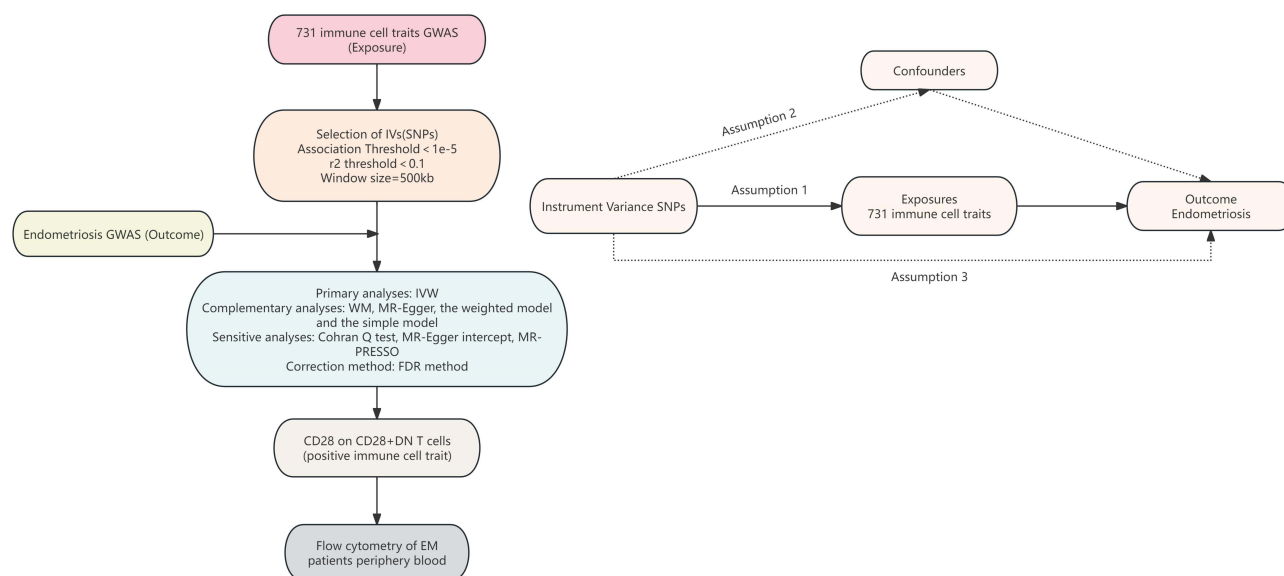
The genome-wide association studies (GWAS) data for 731 immune cell characteristics in this investigation was obtained from the GWAS Catalog (accession numbers GCST0001391 to GCST0002121), which includes four features of seven peripheral blood immune cell populations. The seven immune cell populations include B cells, dendritic cells, T cells at the mature stage, monocytes, myeloid cells, TBNK (T cells, B cells, natural killer cells), and regulatory T cells. The four characteristics were median fluorescence intensity (MFI, n=389), absolute cell count (AC, n=118), relative cell count (RC, n=192), and morphological parameters (MP, n=32).<sup>13</sup> ([Supplementary materials](#)). The GWAS summary statistics for EM were obtained from FinnGen Consortium R9. The study included 15088 cases and 107564 controls, and the sample was all Finnish women.<sup>14</sup> Specific information about the exposure and outcome data is presented in [Table 1](#).

### Selection of Instrumental Variables (IVs)

In order to satisfy the correlation hypothesis of Mendelian assumption, we set the levels of significance for immune traits and cytokines to 1e-5 and use PLINK (version 1.90) to exclude the chain imbalance effect of single nucleotide polymorphisms (SNPs) within 500kb range, with the associated coefficient  $r^2$  threshold setting to 0.1.<sup>15</sup> Subsequently, MR-Egger intercept and MR-PRESSO method were utilized to identify and rule out multi-effectiveness abnormalities.<sup>16</sup>

### Mendelian Randomization

Our MR analysis was implemented in R 4.2 software via R package “TwoSampleMR” (version 0.1.5.6).



**Figure 1** The entire procedure of our study: Assumption1: The correlation hypothesis: genetic variation is directly related to exposure; Assumption2: The Independence hypothesis: there is no connection between the genetic variant and the possible confounders between exposure and outcome; Assumption3: The exclusive hypothesis: the genetic variation will not affect the outcome by means other than exposure.

**Abbreviations:** GWAS, Genome-Wide Association Study; IVs, instrumental variables; SNPs, single nucleotide polymorphisms; WM, the weighted median; MR, Mendelian randomization; EM, endometriosis.

MR was employed to examine the causal relationship between the two exposures and outcomes, including inverse variance weighting (IVW), the weighted median (WM), MR-Egger, the weighted model, and the simple model. IVW is used as the primary method for judging causal effects which provide the highest precision and unbiased causal estimates.<sup>17</sup> The other four analytical methods serve as auxiliary methods to ensure the robustness of the results.<sup>18</sup> Subsequently, we performed a sensitivity analysis using Cochran's Q based on IVW to assess heterogeneity ( $>0.05$  was considered nonsignificant), and we used the funnel plot to judge the reliability of the heterogeneity results.<sup>19</sup>

To satisfy the Mendelian randomization exclusivity hypothesis, we used MR-Egger intercept and MR-PRESSO method to test for horizontal pleiotropy.<sup>16</sup> Considering the presence of false positives, we corrected the P-values for the positives. We utilized the false discovery rate (FDR) method, where  $P_{FDR} < 0.05$  was considered significantly causal and  $P_{FDR} < 0.2$  was considered suggestive causal.<sup>20</sup>

## Flow Cytometry of EM Patient's Peripheral Blood

The expression levels of *CD28 on DN T cells* in peripheral blood of EM patients using flow cytometry. We recruited a total of 6 patients from Anhui Women and Children's Medical Center between April 2024 and June 2024. The EM group included three hospitalized patients with EM confirmed by laparoscopy and histology, and the control group

**Table 1** Details of GWAS Summary Data

| GWAS data                    | Population | Source                                                                                                                                                                                                                                 | Ncase | Ncontrol |
|------------------------------|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|----------|
| 731 immune cell traits       | Sardinians | Valeria Orrù et al<br>( <a href="https://www.ebi.ac.uk/gwas/publications/27989323">https://www.ebi.ac.uk/gwas/publications/27989323</a> )                                                                                              | 3757  | —        |
| finngen_R9_N14_ENDOMETRIOSIS | European   | FinnGenR9<br>( <a href="https://storage.googleapis.com/finngen-public-data-r9/summary_stats/finngen_R9_N14_ENDOMETRIOSIS.gz">https://storage.googleapis.com/finngen-public-data-r9/summary_stats/finngen_R9_N14_ENDOMETRIOSIS.gz</a> ) | 15088 | 107,564  |

**Table 2** Specific Details About the Samples

| Characteristic           | Group                                 |                                       |
|--------------------------|---------------------------------------|---------------------------------------|
|                          | Endometriosis (n=3)                   | Control (n=3)                         |
| Age (years)              | 44.5±2.5                              | 42.5±1.5                              |
| BMI (kg/m <sup>2</sup> ) | 21.5±3.3                              | 22.3±3.1                              |
| Menstrual cycling        | Regularly (6–7 days every 28-30 days) | Regularly (6–7 days every 28-30 days) |
| Dysmenorrhea             | 3                                     | 0                                     |

contained three hospitalized patients whose imaging examination and tumor markers did not suggest EM.<sup>21</sup> Neither group had any other comorbidities and did not receive any treatment at the time of blood collection. Collection of peripheral blood samples from both groups was performed by the patient at Anhui Women and Children’s Medical Center before surgery. This study has been approved by the Ethics Committee of Anhui Women and Children’s Medical Center. Specific details about the samples can be found in [Table 2](#).

5–10 mL of peripheral blood was taken and kept at 2–8°C. The specimens were gently mixed with antibodies and incubated in the dark for 15–30 minutes. Subsequently, 1.5 mL of red cell lysate was added for hemolysis and left for 10 minutes. The mixture was centrifuged at 1000 revolutions per minute for 5 minutes, and the supernatant was removed before being rinsed with phosphate buffered saline (PBS). The resulting product underwent another centrifugation at 1000 revolutions per minute for 5 minutes. The sample was then suspended in 300 microliters of PBS with moderate agitation for analysis.<sup>22</sup> Surface marker expression was measured using Kaluza Analysis on a BD FACSCanto (10-color) flow cytometer. FSC-H-Line / FSC-A-Line dual parameter scatter was plotted to remove adhesion cells. FSC-A-Line / SSC-A-Log dual parameter scatter was plotted to remove fragments. CD45-A-Log / SSC-A-Log two-parameter scatter was plotted to circle nucleated cells. Utilize antibodies targeting specific subpopulations of lymphocytes to determine the proportion of CD3+CD4-CD8-TCRab+ cells within lymphocytes or CD3+ T lymphocytes, as well as their rate of positive CD28 expression.<sup>23</sup> The reagents used are described in the [Supplementary materials](#).

**Results**

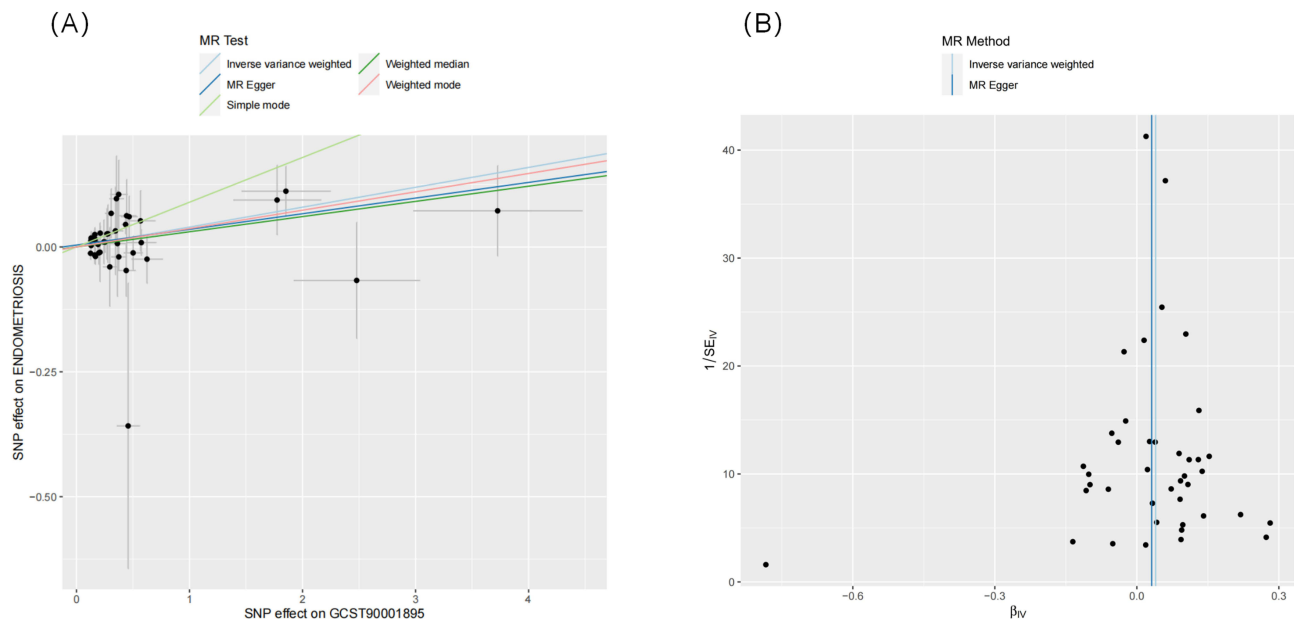
**Exploration of the Causal Relationship Between Immune Traits and EM**

We performed two-sample MR with EM by using 731 immune cell traits as exposure factors ([Supplementary materials](#)). There were no significant differences in immune cell traits when adjusting for multiple tests based on the FDR method ( $P<0.05$ ). At a significance level of 0.20, one immune cell trait with suggestive evidence of association was identified. We discovered that elevated levels of CD28 [*CD28 on CD28 + DN (CD4-CD8-)*] are associated with a higher risk of developing EM ( $\beta =0.040$ , 95% CI =1.02–1.06,  $P =0.00029$ ,  $P_{FDR} = 0.1984$ ). The results of the other two methods were similar: Weighted median (OR =1.031, 95% CI =1.00–1.07,  $P =0.082$ ); MR-Egger (OR = 1.032, 95% CI =1.10–1.06,  $P =0.044$ ) ([Figure 2](#)). Scatter plots and funnel plots indicate the reliability of the results ([Figure 3](#)). The Cochran’s Q test excluded heterogeneity, and both MR-Egger intercept and MR-PRESSO excluded the possibility of horizontal pleiotropy [Table 3](#). It is worth mentioning that some unadjusted low P-value phenotypes, including *IgD on IgD + CD24-* ( $\beta =0.036$ , 95% CI =1.01–1.06,  $P =0.001$ ), *IgD on IgD+ CD38-unsw mem* ( $\beta =0.032$ , 95% CI =1.01–1.05,  $P =0.002$ ), and *CD28 on CD39+ activated Treg* ( $\beta =0.034$ , 95% CI =1.01–1.06,  $P =0.002$ ), may also have had an effect on the occurrence of EM.

| Outcome       | Exposure                    | MR Method                 |  | OR (95%CI)       | P-value  | FDR   |
|---------------|-----------------------------|---------------------------|--|------------------|----------|-------|
| ENDOMETRIOSIS | CD28 on CD28+ DN (CD4-CD8-) | Inverse variance weighted |  | 1.04 (1.02-1.06) | 2.90e-04 | 0.198 |
|               | CD28 on CD28+ DN (CD4-CD8-) | Weighted median           |  | 1.03 (1.00-1.07) | 7.00e-02 |       |
|               | CD28 on CD28+ DN (CD4-CD8-) | MR Egger                  |  | 1.03 (1.00-1.06) | 4.41e-02 |       |
|               | CD28 on CD28+ DN (CD4-CD8-) | Simple mode               |  | 1.09 (1.03-1.16) | 3.77e-03 |       |
|               | CD28 on CD28+ DN (CD4-CD8-) | Weighted mode             |  | 1.04 (1.00-1.07) | 3.19e-02 |       |

**Figure 2** Positive MR results of the causal role between immune cell traits and endometriosis after corrected P-values.

**Abbreviations:** OR, odds ratio; FDR, false discovery rate.



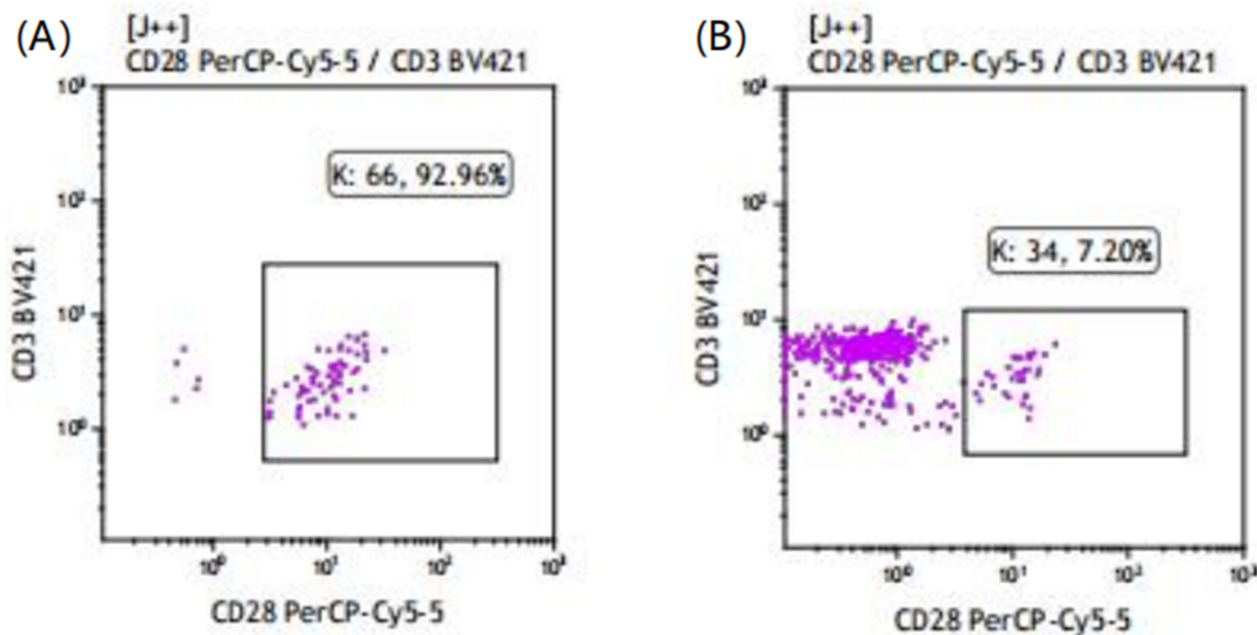
**Figure 3** Scatter plots and funnel plots illustrating the causal associations between immune cell traits and endometriosis: All scatter plots and funnel plots verify the robustness of MR results. **(A)** Scatter plots of CD28 on CD28+ DN (CD4-CD8-) on ENDOMETRIOSIS; **(B)** Funnel plots of CD28 on CD28+ DN (CD4-CD8-) on ENDOMETRIOSIS.

## Flow Cytometry of Peripheral Blood Analysis

The expression levels of *CD28 on DN T* cells were determined using flow cytometry. Compared with the control group, the *CD28 on DN T* cells expression was significantly higher in the peripheral blood of EM patients. As shown in [Figure 4](#), the expression level of *CD28 on DN T cells* was as high as 92.96%, compared to only 7.2% in the control group.

**Table 3** MR Analysis Results of Causal Relationships Between Immune Cell Traits and Endometriosis

| Exposure                    | SNPs | Method          | OR (95% CI)                                           | P                          | Heterogeneity |      |        | Horizontal pleiotropy | MR-PRESSO |
|-----------------------------|------|-----------------|-------------------------------------------------------|----------------------------|---------------|------|--------|-----------------------|-----------|
|                             |      |                 |                                                       |                            | Q             | Q_df | P      | Intercept P           | P         |
| CD28on CD28 + DN (CD4-CD8-) | 41   | IVW<br>WM<br>ME | 1.04(1.02-1.06)<br>1.03(1.00-1.07)<br>1.03(1.00-1.06) | 0.0003<br>0.0818<br>0.0441 | 32.88         | 40   | 0.7804 | 0.4161                | 0.7918    |



**Figure 4** Flow cytometry identified CD28 expression rate of CD3 + CD4-CD8- TCRab + cells and CD3 + T lymphocytes: (A) Endometriosis group; (B) Control group.

## Discussion

Mendelian randomization, a method of statistical analysis used to infer causality based on genetic variables, has been increasingly employed in etiological research recently.<sup>24</sup> This study utilized MR analysis to investigate the causal relationship between 731 immune cell characteristics and EM based on the large sample size of the publicly available GWAS database. Our study identified an immune cell trait with suggestive causality with EM.

Endometriosis is considered a chronic inflammatory disease, and some studies have reported its association with the dysfunction of the lymphocyte population and the cytotoxicity of natural killer cells.<sup>25</sup> DN T cells make up only a small fraction of T lymphocytes in the bloodstream, with a phenotype characterized by the lack of CD4 and CD8 coreceptors and the expression of the  $\gamma\delta$  or  $\alpha\beta$  T cell receptor (TCR).<sup>26</sup> One experimental study verified the tilt of the CD4 Th1/Th2 imbalance in the endometriotic environment of mouse. CD4 Th1 cells inhibit the progression of EM by producing IFN- $\gamma$  and IL-2, while inhibiting the secretion of IL-4 and IL-10 by CD4+ Th2 cells inhibits the development of ectopic lesions in mice.<sup>6</sup> Previous studies found that helper T cell (Th)-like DN T cells, release cytokines, such as interleukin (IL)-4, IL-17, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon  $\gamma$  (IFN- $\gamma$ ). These cytokines enable helper T cell (Th)-like DN T cells to perform functions comparable to those of CD4+ Th cells in the context of infection and autoimmune diseases.<sup>27</sup> This indicates that DNT cells may participate in the pro-inflammatory immune response and cellular immunity through multiple inflammatory factors, tilting the CD4 Th1/Th2 imbalance in the endometriotic environment toward Th2, thus leading to the development of EM.<sup>28</sup>

Cluster of differentiation 28 (CD28) acts as a costimulatory signal in T cells that regulates the function of effector T cells and Treg cells, and it also functions as an induced T cell coreceptor. The reduction in NK cytotoxicity has been shown to be associated with the pathogenesis and progression of EM.<sup>6</sup> Moreover, it has been suggested that the reduced expression of CD28 may inhibit the activation of NK cells and the function of the T cell response in the endometriotic environment.<sup>29</sup> This is different from the result that the risk of EM increased with the expression level of *CD28 on DN T cells* based on MR analysis. We considered the possibility that the expression of *CD28 on DN T cells* changed the direction of its effect on EM. Our clinical samples flow cytometry demonstrated that EM patients indeed showed significant differential expression of *CD28 on DN T cells*, which confirmed the reliability of our results.

As a diagnosis and treatment mode of EM, laparoscopic surgery is clearer than imaging examination and serological examination, and can significantly improve pain symptoms and enhance fertility. Nevertheless, given that the efficacy of



laparoscopic surgery partially relies on the physician's visual assessment, instances of missed diagnoses and recurrences do persist.<sup>30</sup> Therefore, in the past 10 years, an increasing number of studies have discussed in depth the feasibility, efficacy and safety of robotic-assisted surgery (RAS) for EM, and the results are non-inferior to laparoscopic surgery. However, a recent study found that although the robot-assisted approach was effective in restoring ureteral function and resolving symptoms in patients with ureteric EM, the recurrence rate of surgery has not improved.<sup>31</sup> A retrospective cross-sectional study of 142 EM patients with initially diagnosed negative laparoscopic peritoneal pathology revealed that 39% had occult microscopic endometriosis.<sup>32</sup> Therefore, it is particularly important to identify a routine, cost-effective, and non-invasive screening method prior to abdominal exploration. A study involving 88 women clinically advised to undergo laparoscopic surgery for EM collected their blood samples to measure serum cancer antigen 125 (CA125), platelet levels, and total and differential leukocyte counts to assess inflammation. The findings indicated that elevated Systemic Inflammatory Response Index (SIRI) and Neutrophil-to-Lymphocyte Ratio (NLR) were significant predictors of positive laparoscopy results, with high serum CA125 and NLR being the most critical predictors for severe EM (stages III–IV) during laparoscopy.<sup>33</sup> The latest study proposes that upregulated biomarkers can be selected as specific targets in the surgical treatment of EM, using targeted fluorescent tracers for intraoperative tissue identification, and intravenous or local administration depending on the location of the target tissue. Fluorescence-guided surgery (FGS) can improve the detection of endometriosis lesions and delineate the resection plane for a more precise and safe complete excision, thereby reducing the recurrence rate.<sup>34</sup> Similarly, consideration of CD28 on DN T cells as a peripheral predictor or a specific target for FGS of EM warrants further exploration. Our study revealed that EM patients exhibit increased expression levels of CD28 on DN T cells, which may serve as a potential new inflammatory marker and specific target, offering a novel avenue for the diagnosis and treatment of EM.

Our results are robust since the pleiotropy and heterogeneity were not founded. However, there are still some limitations. The GWAS data samples for the exposure factor in this study were not stratified by gender, whereas the GWAS samples for the outcome consisted only of females. This lack of gender stratification could cause bias in the results, despite the fact that many articles with the same condition have been published.<sup>35</sup> We did not utilize the Pheno Scanner to extract the single SNPs from the positive results in order to eliminate any biased SNPs.<sup>36</sup> Ultimately, the samples we obtained were from European populations, therefore, additional verification is necessary to determine if this conclusion is applicable to Asian communities.<sup>37</sup> The findings of our study indicate that a higher level of CD28 on DN T cells is associated with a higher risk of EM onset. Flow cytometry experiments support this conclusion, but the clinical sample size was small. Future larger prospective studies are needed to clarify the clinical effectiveness of CD28 in the diagnosis and treatment of EM.

## Conclusion

In this study, we investigated the causal relationship between peripheral immune cell traits and EM by using two-sample Mendelian randomization (MR) method. The results were confirmed using clinical samples, but larger-scale prospective studies are needed to support clinical effectiveness. Our results contribute to understanding the role of immune function in EM genetics and biology, which may serve as a potential new inflammatory marker and specific target, offering a novel avenue for the diagnosis and treatment of EM.

## Data Sharing Statement

The datasets analyzed for this study can be found in the GWAS catalog and FinnGenR9 release respectively, and the download address can be found in [Table 1](#) and [Supplementary materials](#). The data used and/or analyzed in the present research are available from the corresponding author on reasonable request.

## Ethics Approval

Anhui Women and Children's Medical Center Ethics Committee approved the study protocol (Ethical number: YYLL20240424-LW-LL-05-1.0). The experiment has passed the audit of the Chinese Clinical Trial Registry (Registration number: ChiCTR2400088899). All participants were informed of the study procedures and objectives, and this study was conducted after participants signed an informed consent form. This study complied with the

Declaration of Helsinki, adhered to ethical guidelines and ensured that participants' rights and confidentiality were protected.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare no conflicts of interest in this work.

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